

Oleaxonchium olearum gen. et sp. nov. (Nematoda, Dorylaimida) associated with an olive grove in the southern Iberian Peninsula, and new insights into the evolutionary relationships within Belondiridae

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Abstract

A new belondirid, dorylaimid taxon, *Oleaxonchium olearum* **gen. et sp. nov.**, collected from an olive grove in the Andalusia region of Spain, is characterised, including its morphological description, morphometrics, SEM study, and molecular (18S-, 28S-rDNA) analyses. The new genus displays a unique combination of traits that distinguishes it from its closest genera: a rectangular lip region with sclerotized margins when observed in lateral view and visibly hexagonal in face view under SEM; a comparatively long cheilostom with thickened walls at its anterior part; a short isthmus-like section separating both pharyngeal regions; a mono-opistho-ovarian didelphic female genital system without *pars refringens vaginae*; and a short and rounded tail. The new species is characterised by its 2.44–2.87 mm long body, lip region 7–7.5 µm wide, odontostyle 10–10.5 µm long, neck 723–973 µm long, pharyngeal expansion occupying 63–72% of the total neck length, female anterior genital branch 4–6% of body length, tripartite posterior uterus 1.9–2.6 body diameters long, with a short intermediate section bearing sclerotized elements, vulva ($V = 58–61$) a transverse slit, caudal region 29–35 µm long ($c = 74–89$, $c' = 0.9–1.1$), and male unknown. As derived from an integrative approach combining morphological and molecular data, the new genus is close to *Metaxonchium*, the polyphyly of Belondiridae is confirmed, and support is provided in favour of the monophyly of Axonchiinae.

Key Words

morphology, new taxa, phylogeny, SEM, taxonomy, 18S-, 28S-rDNA

Introduction

Dorylaims, the members of the order Dorylaimida, are probably the most diverse nematode taxon. Being present in any continental (soil and freshwater) habitat and exploiting a wide spectrum of feeding resources, they are regarded as good bioindicators of environmental health (Peña-Santiago 2021). With 36 valid genera and 242 valid species, the family Belondiridae Thorne, 1939 represents almost 10 percent of all the dorylaims (Andrássy 2009). This family currently includes three subfamilies, one of which, Belondirinae Thorne, 1939, is less diverse and more frequent in temperate areas.

The free-living dorylaimid fauna of olive groves is poorly explored in spite of the fact that this culture is the most prominent and most socioeconomically important fruit tree in Europe, especially across the Mediterranean Basin (Vilar and Pereira 2018). Actually, at present, only eight species, eight genera, and six families were previously found in dwelling olive orchards (unpublished data). Representatives of Belondirinae are scarce in the Iberian fauna, with four species hitherto recorded, mainly in natural areas (Jiménez-Guirado et al. 2007).

A belondirid population was collected in the course of a nematological survey conducted to study the free-living fauna inhabiting olive soils in the framework of the

project Soil O-Live (EU Horizon Programme Grant No. 101091255). Its morphological and molecular study revealed that it represented a new generic taxon. Thus, this contribution aims to describe its morphological pattern, obtain its molecular characterization, and discuss its evolutionary relationships.

Materials and methods

Nematodes and their morphological and morphometrical study

A total of 12 female specimens, found in the soils of an olive grove named “La Capilla” (Spain, Málaga province, Antequera municipality), were available to study. Nematodes were extracted by centrifugation (CDEA 2015; based on Jenkins 1964) and/or with Baermann’s funnels following the protocol by Flegg (1967), somewhat modified, killed by heat, fixed in 4% formaldehyde, preserved in anhydrous glycerin according to Siddiqi’s (1964) method, mounted on permanent glass slides that were sealed with paraffin, and measured and photographed using an Eclipse 80i microscope (Nikon) equipped with differential interference contrast optics, a drawing tube (*camera lucida*), and a DS digital camera. Morphometrics include Demanian indices and other measurements and ratios; some of them are presented in a separate table, while others form part of the literal description of species. Two specimens preserved in glycerin were selected for observation with a SEM, according to Abolafia (2015). The nematodes were hydrated in distilled water, dehydrated in a graded ethanol-acetone series, critical point dried, coated with gold, and observed with a Zeiss Merlin microscope (5 kV).

Molecular study

For molecular analyses, single specimens were temporarily mounted in a drop of 1 M sodium chloride containing glass beads. This was followed by DNA extraction from single individuals, as described by Archidona-Yuste et al. (2016). The D2–D3 domains were amplified using the D2A (5′–ACAAGTACCGTGAGGGAAAGTTG–3′) and D3B (5′–TCGGAAGGAACCAGCTACTA–3′) primers (De Ley et al. 1999). The portion of 18S rRNA was amplified using primers 988F (5′–CTCAAAGATTAAGCCATGC–3′), 1912R (5′–TTTACGGTCAGAACTAGGG–3′), 1813F (5′–CTGCGTGAGAGGTGAAAT–3′), and 2646R (5′–GCTACCTTGTTACGACTTTT–3′) (Holterman et al. 2006). All polymerase chain reaction (PCR) assays were done according to the conditions described by Archidona-Yuste et al. (op. cit.). The amplified PCR products were purified using ExoSAP-IT (Affimetrix, USB products) and used for direct sequencing on a DNA multicapillary sequencer (Model 3130XL genetic analyser; Applied Biosystems, Foster City, CA, USA), using the BigDye Terminator Sequencing Kit V.3.1 (Applied Biosystems, Foster City, CA, USA), at the StabVida

sequencing facilities (Caparica, Portugal). The newly obtained sequences were submitted to the GenBank database under the accession numbers indicated on the phylogenetic trees.

Phylogenetic analyses

For phylogenetic relationships, analysis was based on 18S and 28S rDNA fragments. The obtained sequences were manually edited using Chromas 2.6.6 (Technelysium) and aligned with other rDNA sequences available in GenBank using the ClustalW alignment tool implemented in MEGA7 (Kumar et al. 2016). Poorly aligned regions at extremes were removed from the alignments using MEGA7. The best-fit model of nucleotide substitution used for the phylogenetic analysis was statistically selected using jModelTest 2.1.10 (Darriba et al. 2012). The phylogenetic tree was generated with the Bayesian inference method using MrBayes 3.2.6 (Ronquist et al. 2012). The analysis under the general time reversible plus invariant sites plus gamma distribution (GTR + I + G) model was initiated with a random starting tree and run with the Markov chain Monte Carlo (MCMC) (Larget and Simon 1999) for 1×10^6 generations. The tree was visualised and saved with FigTree 1.4.4 (Rambaut 2018).

Results

Description of new taxa

Oleaxonchium gen. nov.

<https://zoobank.org/D5B8C148-9476-4684-AC45-255492E8F9A6>

Diagnosis. Belondiridae, Belondirinae. Medium-sized nematodes, 2.44–2.87 mm long. Cuticle dorylaimid, thick, two-layered. Body pores button-like. Lip region narrower than the adjoining body, rectangular in lateral view, with distinctly sclerotized margins, totally fused lips, and simple, pore-like cephalic papillae. Amphid cup-like, with large aperture. Cheilostom a relatively long truncate cone with thickened walls at its anterior (perioral) part. Odontostyle small, robust, slightly fusiform, with short aperture, up to one-third of its length. Guiding ring simple, refractive. Odontophore linear, lacking any differentiation. Pharynx entirely muscular, with both regions separated by a short, isthmus-like narrowing; basal expansion occupying *ca* two-thirds of the total neck length, surrounded by a conspicuous muscular sheath. Female genital system mono-opistho-ovarian, didelphic, without *pars refringens vaginae*, well-developed *pars distalis vaginae*, and transverse vulva. Caudal region short and rounded. Male unknown.

Type and only species. *Oleaxonchium olearum* sp. nov.

Etymology. The genus name is derived from *Olea europaea* L., the scientific name of olive, as the type species was found associated with this typical Mediterranean fruit tree, and *Axonchium*, a very close taxon.

***Oleaxonchium olearum* gen. et sp. nov.**
<https://zoobank.org/244ABA7C-4365-4FE8-9340-F765726A3811>
Figs 1–4

Material examined. Twelve females from one location are in good condition for preservation.
Morphometrics. See Table 1.

Table 1. Main morphometrics of *Oleaxonchium olearum* gen. et sp. nov. from Spain. Measurements in μm except L in mm are in the form of average \pm sd (range).

Character n	Holotype	Paratypes
	♀	11 ♀♀
L	2.87	2.61 \pm 0.12 (2.44–2.87)
a	60	56.6 \pm 3.3 (53–64)
b	2.9	3.1 \pm 0.3 (2.6–3.5)
c	84	81.8 \pm 5.4 (74–89)
V	59	58.6 \pm 0.7 (58–61)
c'	1.0	1.0 \pm 0.1 (0.9–1.1)
Lip region diameter	7.5	7.03 \pm 0.3 (7.0–7.5)
Odontostyle length	10	10.1 \pm 0.2 (10–10.5)
Odontophore length	16	15.7 \pm 0.4 (15–16)
Neck length	973	894 \pm 86 (723–973)
Pharyngeal expansion length	670	566 \pm 79 (458–679)
Body diameter at neck base	47	44.5 \pm 1.7 (42–47)
mid-body	48	46.2 \pm 1.6 (44–48)
anus	34	33.6 \pm 0.9 (32–35)
Distance vulva – anterior end	1680	1530 \pm 79 (1419–1680)
Prerectum length	392	361 \pm 25 (326–392)
Rectum length	35	33.4 \pm 2.4 (29–37)
Tail length	34	32 \pm 1.9 (29–35)

Description. Female. Very slender and medium-sized nematodes. Body cylindrical, conspicuously tapering towards the anterior end, much less so towards the posterior one as the tail is short and rounded. Upon fixation, habitus curved ventrad to a more or less open C shape. Cuticle smooth, two-layered, 0.5–1 μm thick at anterior region, 2.5–4 μm in mid-body, and 8–11 μm on tail, consisting of a thinner outer layer and a much thicker inner layer bearing radial striation, especially appreciable at caudal region. Large cervical lacunae are present. Lateral chord very narrow, 4–10 μm wide, occupying up to one-fifth (9–21%) of midbody diameter, bearing abundant elliptical gland bodies, more perceptible at posterior body region. Lateral pores button-like, when observed with SEM. Lip region almost rectangular in lateral view, visibly narrower than the adjoining body, 1.8–2.1 times wider than high, and *ca* one-sixth (15–17%) of body diameter at neck base, its anterior and lateral margins sclerotized and somewhat refractive, with hardly visible labial and cephalic papillae. SEM observations: lip region almost octagonal when observed in face view, with totally fused lips; its margin slightly elevated, marking off a somewhat sunken area; oral field comparatively small as inner labial papillae are almost equidistant between the oral aperture and the marging, far from the outer labial papillae; labial papillae button-like, delimited by a small circular incisure, with a distinct pore at their center, cephalic papillae unusually simple, small pore-like. Amphid fovea cup-like, its aperture 6.5–7.5 μm long, almost equal (0.9–1.1 times) to

the lip region width. Cheilostom a truncate cone 11.5–12 μm long, 1.6–1.7 times as long as the lip region width, with thin walls that distinctly thicken at its anterior (perioral) end. Odontostyle slightly fusiform, 5–7 times as long as wide, longer (1.3–1.5 times) than lip region width, 0.35–0.42% of body length, its aperture 2–3 μm or less than one-third (20–30%) of total length. Guiding ring simple, distinct. Odontophore rod-like, 1.4–1.6 times the odontostyle, bearing very weak thickenings at its base. Pharynx very long, consisting of a slender anterior region, a very short narrower (isthmus-like) intermediate section, and a large, strongly muscular posterior expansion 18–38 times as long as wide, 10–15 times longer than body diameter at neck base, which occupies *ca* two-thirds (63–72%) of the total neck length, and appears enveloped by a strong spiral muscular sheath with almost strait bands; gland nuclei obscure in the specimens examined. Nerve ring situated at 147–178 μm , or 17–22% of the total neck length from the anterior end. Pharyngo-intestinal junction consisting of a cylindrical, tongue-like, 20–27 \times 6.5–11 μm cardia, almost totally surrounded by intestinal tissue. Genital system mono-opistho-ovarian, didelphic: anterior branch reduced, 102–165 μm long or 4–6% of body length, consisting of a tube-like uterus, a narrowing, probably a very weak sphincter, and a distal cell mass 20–24 μm long, certainly representing a vestigial oviduct and/or ovary; posterior branch well-developed, 190–303 μm long or 8–11% of body length, with a variably large ovary 56–146 μm long, often not reaching the sphincter, bearing oocytes first in several rows and then in a single row, oviduct 72–116 μm long or 1.6–2.6 body diameters that consists of a slender distal region made of prismatic cells and a proximal *pars dilatata* with visible lumen inside, sphincter present but not especially distinct between oviduct and uterus, uterus 87–122 μm long or 1.9–2.6 body diameters (its length however should be taken with caution as it always appears convoluted), tripartite as it consists of a longer proximal region with wider lumen, a shorter intermediate section that bears refractive elements (Z-like structure), and a dilate, almost spherical, distal part. Vagina 22–27 μm long, extending inwards to often more than one-half (48–60%) of body diameter: *pars proximalis* 11–16 \times 8–17 μm and encircled by moderately developed musculature, *pars distalis* 9–12 μm long. Vulva a transverse slit *ca* 10 μm long. Prerectum 9.6–12.2, rectum 0.9–1.1 anal body diameters long. Anus a visibly arched transverse slit *ca* 4 μm long. Caudal region short and rounded, its inner core reaching 61–77% of tail length, caudal pores two pairs, sublateral, at the middle of tail.

Male. Unknown.

Molecular characterization. After sequencing and editing, ten sequences of type specimens were obtained for phylogenetic analyses. Six 18S rDNA sequences, which were 1653, 1696, 1705, and 1709 (three sequences) bp length (acc. PP956610–PP956615, respectively), presented 1651 bp in common, and a Blast search showed that they were 98.97% similar to those of *Axonchium* sp. (acc. MG921264 and OQ946544). Four 28S rDNA sequences, which were 728, 739, 742, and 745 bp length (acc. PP956616–PP956619, respectively), presented 720 bp in common, and a Blast search showed that they were 93.34%

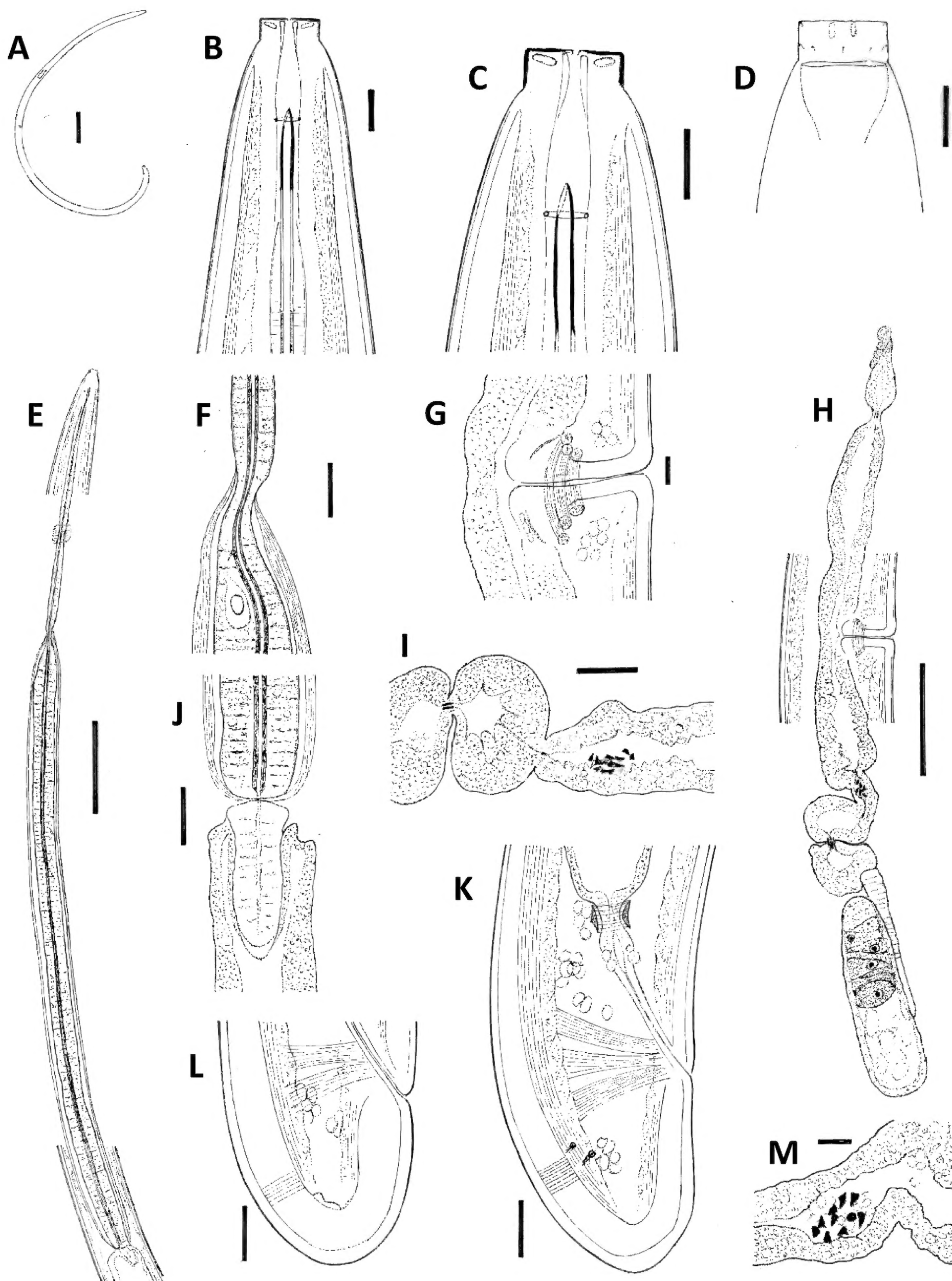


Figure 1. *Oleaxonchium olearum* gen. et sp. nov. (female). **A.** Entire; **B-D.** Anterior body region in lateral median (**B**, **C**) and lateral surface (**D**) views; **E.** Neck region; **F.** Isthmus-like narrowing between both pharyngeal sections; **G.** Vagina; **H.** Genital system; **I.** Oviduct-uterus junction; **J.** Pharyngo-intestinal junction; **K**, **L.** Caudal region; **M.** Detail of uterine Z-like structure. Scale bars: 200 µm (**A**); 5 µm (**B-D**, **G**, **M**); 100 µm (**E**); 10 µm (**F**, **I-L**). 50 µm (**H**).

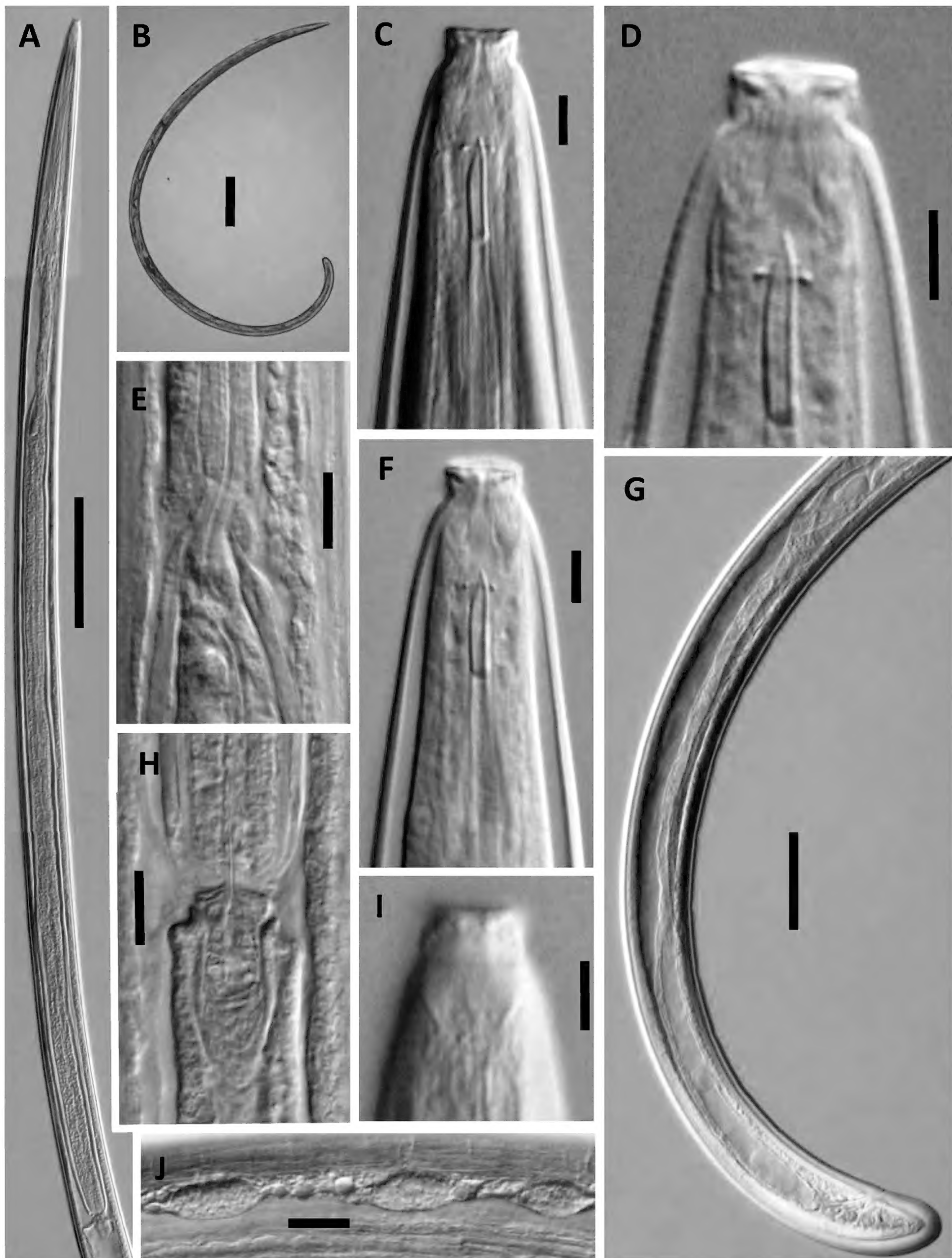


Figure 2. *Oleaxonchium olearum* gen. et sp. nov. (female, general morphology, LM). **A.** Neck region; **B.** Entire; **C, D, F, I.** Anterior body region in lateral median (**C, D, F**) and lateral surface (**I**) views; **E.** Isthmus-like narrowing between both pharyngeal sections; **G.** Posterior body region; **H.** Pharyngo-intestinal junction; **J.** Gland-like bodies at the lateral chord. Scale bars: 100 µm (**A**); 200 µm (**B**); 5 µm (**C, D, F, I**); 50 µm (**G**); 10 µm (**E, H, J**).



Figure 3. *Oleaxonchium olearum* gen. et sp. nov. (female, genital system, and caudal region, LM). **A.** Genital system; **B.** Anterior genital branch; **C.** Oviduct-uterus junction; **D, E.** Vagina; **F, G.** Rectum and caudal region; **H.** Detail of uterine Z-like structure. Scale bars: 50 µm (**A, B**); 10 µm (**C, F, G**); 5 µm (**D, E, H**).

similar to those of *Metaxonchium giennense* (acc. OQ473053–OQ473054), 91.14% to those of *Metaxonchium magnum* (OQ099691, OQ099693), and 88.86% to that of *Axonchium* sp. (ON927906).

Diagnosis. The new species is characterised by its 2.44–2.87 mm long body, lip region 7–7.5 µm wide, odontostyle 10–10.5 µm long, neck 723–973 µm long, pharyngeal expansion occupying 63–72% of the total neck length, female genital system mono-opistho-ovarian, didelphic, with anterior branch occupying 4–6% of body length, posterior uterus 87–122 µm long or 1.9–2.6 body diameters and tripartite, with a short intermediate section bearing refractive elements, vulva ($V = 58\text{--}61$) a transverse slit, caudal region short

and rounded (29–35 µm, $c = 74\text{--}89$, $c' = 0.9\text{--}1.1$), and male unknown.

Type locality and habitat. Spain, Málaga province, Antequera municipality, “La Capilla” farm (37.198283, -4.543868, elevation 491 m), where the new species was collected in an olive grove with intensive tillage.

Type material. Female holotype and nine female paratypes were deposited in the nematode collection of the University of Jaén, Spain. Two female paratypes were deposited with the United States Department of Agriculture Nematode Collection (USDANC) at Beltsville, MD, USA.

Etymology. The species epithet is the Latin term *olearum* = belonging to or corresponding to olives, as type material was found in an olive grove.

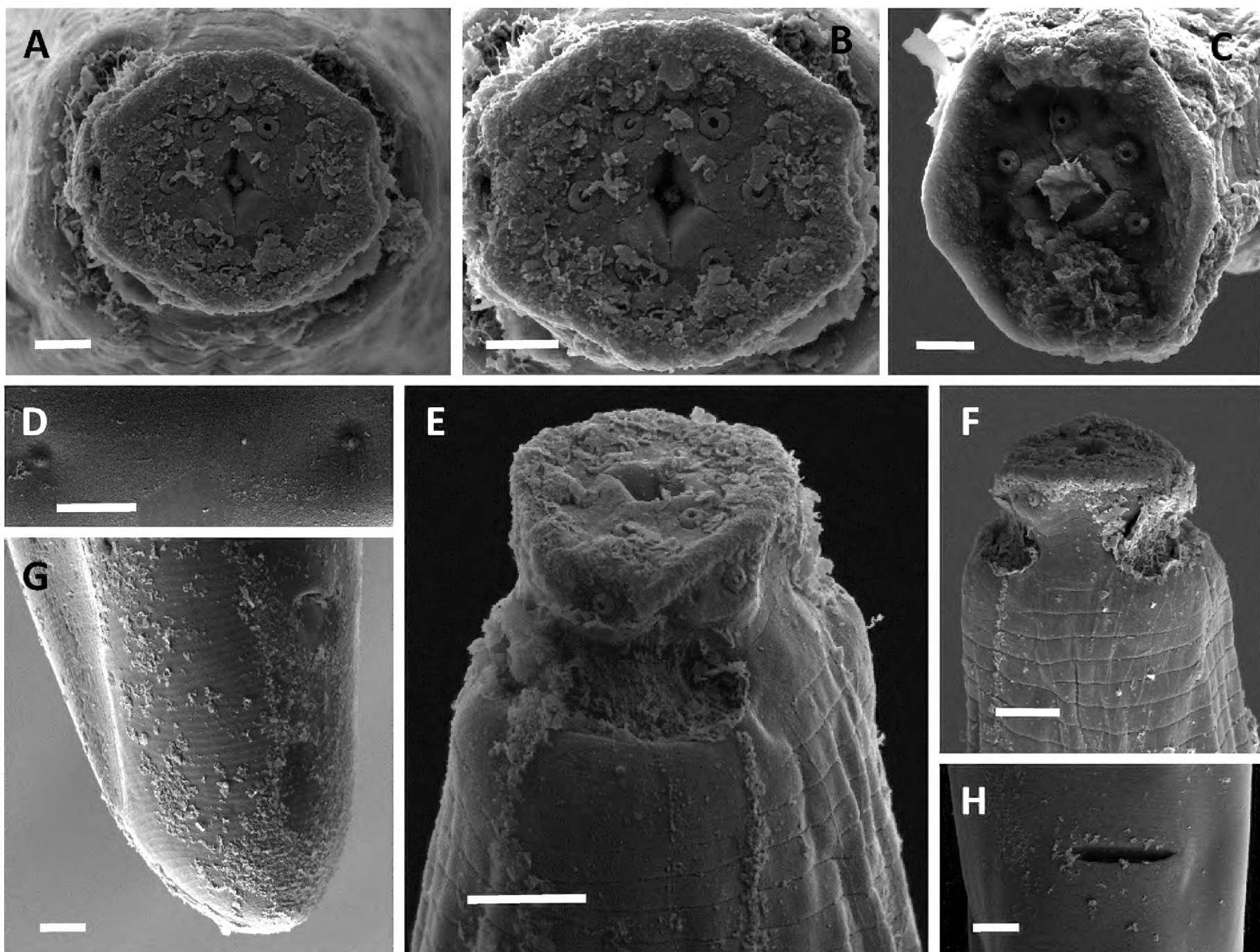


Figure 4. *Oleaxonchium olearum* gen. et sp. nov. (female, SEM). **A–C.** Lip region, in face view; **D.** Lateral pores; **E.** Anterior body region, lateral view; **F.** Anterior body region, ventral view; **G.** Caudal region, sublateral view; **H.** Vulva, ventral view. Scale bars: 1 μm (**A–C**); 5 μm (**D**); 2 μm (**E, F**); 4 μm (**G, H**).

Evolutionary relationships of the new taxon

As derived from morphological data

The general appearance of the new genus is very similar to that found in representatives of the *Axonchium*-like pattern, including comparatively short and slightly fusiform odontostyle, simple odontophore, both pharyngeal sections distinctly separated, very long pharyngeal expansion, mono-opistho-ovarian, didelphic female genital system, and a short and rounded tail (cf. Coomans and Nair 1975). Some of these traits should be interpreted as relevant apomorphies (fusiform odontostyle, very long pharyngeal expansion, mono-opistho-ovarian female genital system), if not autapomorphies (separation of both pharyngeal sections) within the family Belondiridae (cf. Peña-Santiago and Abolafia 2023). Nevertheless, *Oleaxonchium* gen. nov. is easily distinguishable from other *Axonchium*-like genera in the unique aspect of its lip region (rectangular in lateral view, plane and almost octagonal in face view, and with sclerotized margins vs. typically cap-like and without sclerotized margins), which is herein interpreted as an outstanding autapomorphy. The very small cephalic papillae (simple pores) observed in the type species, as well as its button-like lateral pores, represent apomorphic (if not autapomorphic)

features too. Besides, the cheilostom, comparatively long and with thickened walls at its anterior, perioral part, is an apomorphic condition, never recorded in other genera that bear shorter and thin-walled cheilostoms.

In having a short, narrower (isthmus-like) section between both pharyngeal sections, the new genus is close to *Metaxonchium* Coomans & Nair, 1975, but, leaving aside the lip region, both taxa differ in the shape of the vagina, which lacks (vs. bears) *pars refringens* and presents very well-developed (vs. very short) *pars distalis*, respectively. In the absence of *pars refringens vaginae*, *Oleaxonchium* gen. nov. also resembles the genera *Axonchium* Cobb, 1920, and *Syncheilaxonchium* Coomans & Nair, 1975, differing from them in the nature of their pharynx: a slender anterior region separated from the posterior expansion by a short isthmus-like section vs. a well-muscular anterior region separated from the posterior one by a marked constriction (abutting).

As derived from molecular analyses

The results of the analyses of six 18S rDNA and four 28S rDNA sequences (see above) are presented in the trees of Fig. 5 (18S) and Fig. 6 (28S). In both trees, the sequences of the new taxon form maximally supported clades, confirming their identity. Nevertheless,

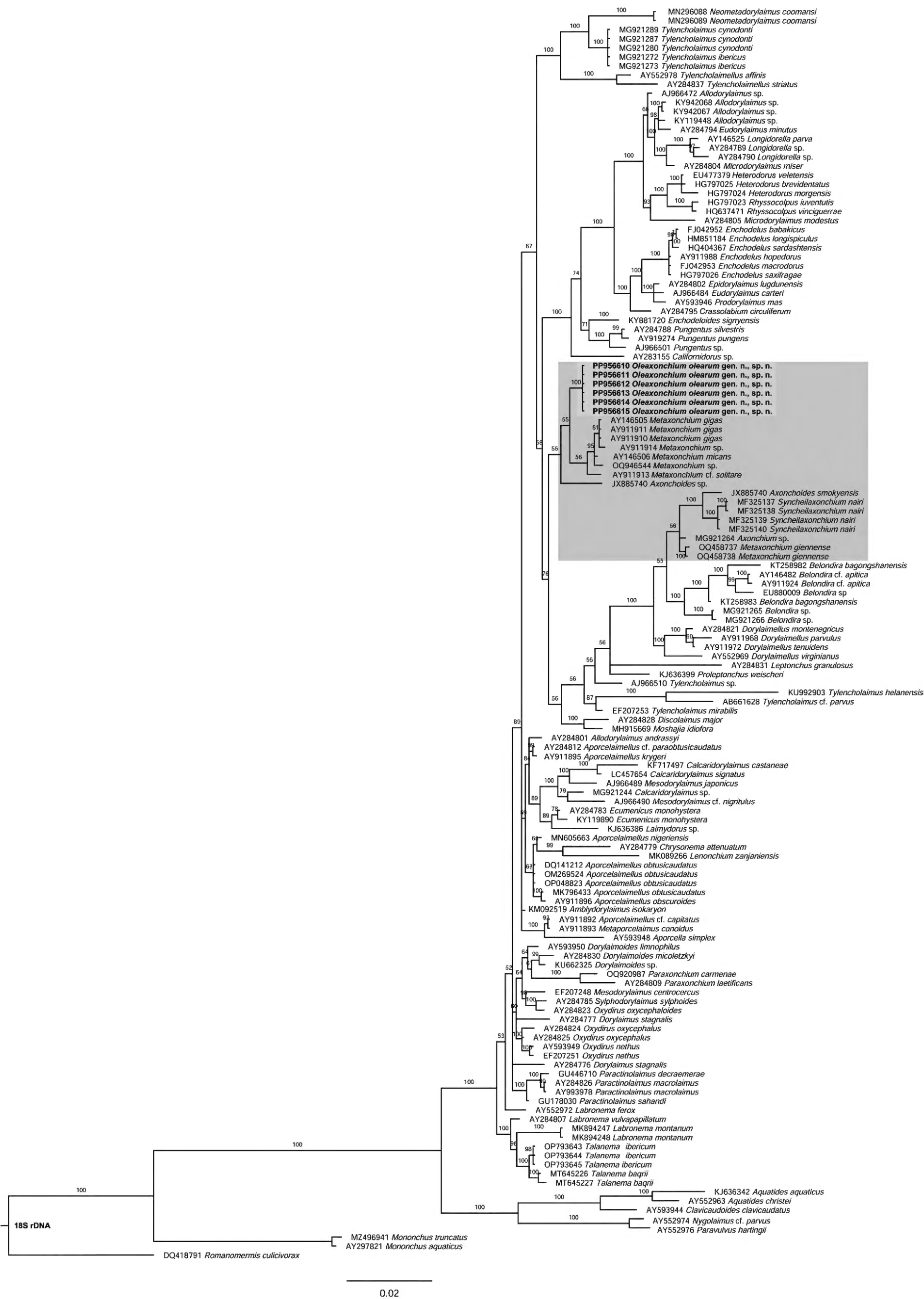
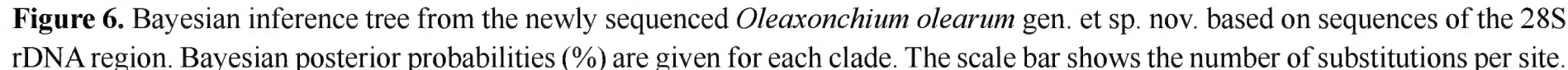


Figure 5. Bayesian inference tree from the newly sequenced *Oleaxonchium olearum* gen. et sp. nov. based on sequences of the 18S rDNA region. Bayesian posterior probabilities (%) are given for each clade. The scale bar shows the number of substitutions per site.



other aspects of its evolutionary relationships significantly differ when the trees are compared.

The 18S tree provides very unsatisfactory branching resolution. On the one hand, the *Oleaxonchium* gen. nov. clade forms part of a very lowly (55%) supported clade, where it appears as the sister group of an also lowly (56%) supported subclade that includes sequences of several *Metaxonchium* species. On the other hand, sequences of *Axonchium*-like genera (*Axonchium*, *Axonchoides*, and *Syncheilaxonchium*) and *M. giennensis* (all of them highlighted in green in the tree) form part of a maximally supported clade with sequences of the genera *Belondira* and *Dorylaimellus*.

The 28S tree offers better resolution as all *Axonchium*-like sequences form a maximally supported clade. Nevertheless, some uncertainties persist about the internal relationships within this clade, mainly due to the apparent polyphyly of *Metaxonchium*, whose sequences form part of three different subclades, namely (new taxon + *M. giennense*), (*Axonchoides smokyensis* + (*M. stenospiculum* + *M. toroense*)), and (*M. magnum* + *Syncheilaxonchium nairi*). Besides, the *Axonchium*-like clade is included in a much larger clade, with *Belondira* sequences not forming part of it.

An integrative approach and general discussion

The singularity of the lip region of *Oleaxonchium* gen. nov., unique within Belondiridae and probably in Dorylaimida, and a recognisable autopomorphy, supports its separation from its closest relatives. The peculiar shape of its body pores and cephalic papillae also represent remarkable differences with other axonchiid genera. Molecular analyses based on both 18S and 28S genes coincide when indicating that there is a closer evolutionary relationship between the new taxon and *Metaxonchium* representatives, but the results significantly differ in the nature and robustness of this relationship when the respective molecular trees are compared. Anyway, *Metaxonchium* is a rather homogeneous genus with a recognisable morphological pattern (Peña-Santiago and Abolafia 2023), easily distinguishable from *Oleaxonchium* gen. nov. (see above).

Present results confirm that the internal evolutionary relationships of Belondiridae remain unsatisfactorily resolved, with morphological and molecular data not always in agreement. First, the polyphyly of the family is once again noted in both 18S and 28S trees, as *Oxydirus* (subfamily Swangeriinae) sequences appear in a separate clade, far from those including other representatives of the group. Second, morphological features and 28S analysis support the validity of Axonchiinae Thorne, 1964, as a monophyletic taxon that should be reinstated (cf. Andrassy 2009). Third, relationships between *Belondira* (subfamily Belondirinae) and axonchiid genera remain obscure, as 18S and 28S analyses did not provide comparable evidence. Once more, further research should be accomplished to clarify these issues.

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